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Research Article

THE OPTIMISED CONDITIONS OF INDUCTION OF RECOMBINANT RIP rMJC15310 ACTIVITY ISOLATED FROM *Mirabilis jalapa* L. LEAVES

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ABSTRACT

Ribosome Inactivating Proteins (RIPs) are compounds isolated from plants with ability to inhibit protein synthesis. The inhibition of protein synthesis is due to inactivation of ribosomal RNA through a site-specific deadenylation mediated by RNA N-glycosidase. Reportedly, RIPs mainly possess wide range of bioactivity including antiviral activity against plant infections. Other activities of RIP were as abortifacien, antivirus and anticancer. This study was aimed to isolate and characterize the optimum conditions for inducing the expression of recombinant RIPs isolated from the leaves of *Mirabilis Jalapa* L. We have been successfully isolated several RIPs and engineered these proteins to be expressed in *E. coli*. These recombinant proteins were obtained by screening cDNA library originated from the mRNA of *Mirabilis jalapa* L leaves, and inserted into pUC19 carrying lacZ gene. The presence of recombinant plasmid was tested by using α-complementation assay. Many RIPs have been isolated from plants and these proteins express enzymatic activity by cutting supercoiled double stranded DNA. One RIP namely rMJC15310 was obtained from this study and the proteins having ~ 8kb in size, cut the supercoiled DNA into linear form at the concentration as low as 5 µg. The ability to cut supercoiled DNA increased on inducing its expression with 0.4% IPTG.

Key words: Ribosome Inactivating Proteins (RIP), IPTG, *Mirabilis jalapa* L., recombinant protein

INTRODUCTION

Ribosome Inactivating Proteins (RIPs) are toxic protein compounds isolated from plants with ability to inhibit protein synthesis. The inhibition of protein synthesis is due to inactivation of ribosomal RNA through a site-specific deadenylation mediated by RNA N-glycosidase (Endo *et al.*, 1987, Endo and Tsurugi 1987). These proteins are also capable of inactivating non-ribosomal nucleic acid substrates and therefore can be referred to as polynucleotide: adenosine glycosidase (Barbieri, 1997). There are three major classes of RIPs based on its primary sequences, type I consisting of single chain protein with molecular weight of 25,000-30,000 Da and type II RIPs having two chains (A and B) connected by disulphide bond with molecular weight of about 60,000 Da (Ling *et al.*, 1994). Type III RIP as that of JIP60 isolated from *Hordeum vulgare*, is a single chain protein carrying amino acid terminal domain, resembling type I RIP, linked to unknown carboxyl-terminal domain. The type II RIPS are synthesized as inactive compounds and converted to the active one through proteolysis mechanisms (Peumans *et al.*, 2001, Stirpe, 2004). Besides RNA glycosidases, RIPs possess enzymatic activity by cleaving supercoiled-double stranded DNA ((Ling *et al.*, 1994, Sismindari *et al.*, 1998).

RIPs has been reported to possess wide range of bioactivity including antiviral activity against plant infections (Wang *et al.*, 1998) and anticancer (Stirpe, 2004). The anticancer properties of a biologically active compound are prefferebly accompanied by the ability to function as anti oxidant, antiproliferation, angiogenesis inhibition, the ability to induce apoptosis as well as to increase the immune system. Since the identification of toxic ricin isolated from the seed of castor bean (*Ricinus comunis*), research has been focused on the

medical and therapeutical application of RIPs (Lin *et al.*, 1970, Endo *et al.*, 1987). Different type of RIPs posses different toxicity against different cell lines. One study reported that RIPs induced apoptosis through the release of cytochrome C from mitocondria dan subsequent activation of caspase 9 and 3 (Qu and Qing, 2004), while others found the phenomenon of caspase 8 and 3 but not 9 activation invitro (Sha, 2010). RIP has been reported to show immunosuppresive capacity in which at low concentration RIP induced IL-1, while at high concentration inhibited the production of IL-1 and cell proliferation (Battelli, 2004). Barbieri e*t al.*, (2006) reported that RIP isolated from *C.moshata* showed antioxidant properties equal to Fe-superoxide dismutase from *E.coli*. The capacity of antioxidant is important for inhibition transformation processes of cancer sel. The angiogenetic effect of RIP on endocyalin-positive neoplasia has been reported on Saporin-conjugated antibody (Roulean *et al.*, 2008). Other activities of RIP as abortifacien, antivirus and cytotoxicity to mammalian cell also have been reported elesewhere (Stripe *et al.*, 1992; Barbieri *at al.*, 1993; Sismindari *et al.*, 2001; Sismindari *et al.*, 2002; Narayanan *et al.*, 2004).

In this study we have been able to screen RIPs recombinant by inserting the cDNA library of RIPs from *Mirabilis jalapa* L leaves to *E.coli*. Few candidates of bioactive RIPs have been obtained and further characterised. One of the candidate namely RIP rMJC15310 was shown to cut supercoiled DNA and the expression was increased upon induction with isopropylthiogalactoside (IPTG). This article reports the optimum conditions for optimizing the production of RIP rMJC15310 in culture.

METHODOLOGY

Materials

Red flowering *Mirabilis jalapa* leaves was collection of LPPT UGM which has been characterised by Pharmaceutical Biology Department, Faculty of Pharmacy, UGM. The sample was collected from young plant which has not been producing fruit. pUC19 was the collection of Integrated Research and Services Laboratory (LPPT) UGM. All the chemicals used in this study were supplied from Sigma, otherwise was stated.

White colony analysis (alpha complementtation assay).

White colony was grown in ampicilin containing LB media at 37°C overnight. Cell was harvested dan sentrifugated. The pellet was washed with 5 mM phosphate buffer pH 6.5 prior to sonication using the same cold buffer. The extract was centrifuged at 2000 g 10 minutes at 4°C and the total protein concentration was analysed using Biuret method.

Cleavage of supercoiled DNA by RIPs

Three µg DNA plasmid pUC19 was mixed with 1 µL Tris buffer containing Tris-HCl 50mM, pH 8.0, $MgCl_2$ 100mM, NaCl 100 mM. The recombinant protein at certain concentrations were added and equilibrated at total volume of 10 µL with water. The mixtures were incubated at 30°C for 1 h. Two µL loading buffer was added dan the DNA was analysed using agarose gel electrophoresis (Ling *et al.*, 1994).

Plasmid isolation

E.coli carrying recombinant protein rMJC15310 were grown in 5 mL LB media containing ampicilin at 37°C overnight. The culture was centrifuged and the cells were resuspended with 100 µL TGE buffer (25mM Tris-Cl pH 8.0, 50 mM glucoce and 10 mM EDTA), and incubated at RT for 5 minutes. 200 µl NaOH containing 1% SDS was added and incubated on ice for 5 minutes. 150 µL 3M.

Potassium acetate solution at pH 4.8 was added on ice for further 5 minutes and the mixture was centrifuged. The supernatant was extracted with 500 µL phenol-chloroform and DNA was precipitated using 50 µL 3M natrium acetate and 1 mL ethanol. The DNA pellet was washed with 70% ethanol and air-dried. DNA was solubilised in 50 µL TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA).

Agarose gel electrophoresis

DNA was loaded on 0.8% agarose gel in TAE buffer (40 mM Tris, 1 mM EDTA, 10 mM acetic acid pH 8.0), containing 0.5 µg/mL ethidium bromide. TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) was used as running buffer and the sample was run at 70 Volt.

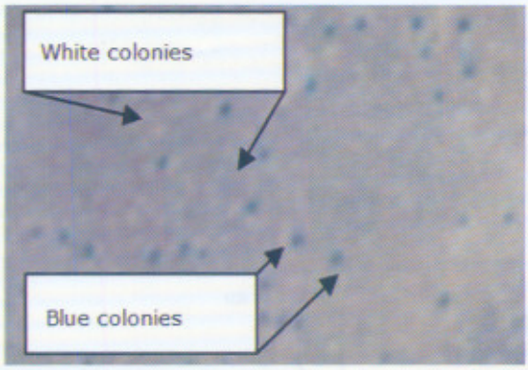

Figure 1. Selection for white colonies of *E.coli* transfected with plasmids pUC19 containing cDNA library inserts.

RESULTS AND DISCUSSION

This study was aimed to isolate and characterize the optimum conditions for inducing the expression of recombinant RIPs isolated from the leaves of *Mirabilis jalapa* L. We have been successfully isolated several RIPs and engineered these proteins to be expressed in *E. coli*. These recombinant proteins were obtained by screening cDNA library originated from the mRNA of *Mirabilis jalapa* L leaves, and inserted into pUC19. In this study we used pUC19 carrying Ampicillin resistance gene and *lacZ* gene under the control of lac promoter. This antibiotic resistant gene is commonly used in method to identify positive transformation, by growing the bacteria in media containing antibiotic (Sambrook *et al.*, 1989).The presence of recombinant plasmid was tested by using α-complementation assay in which the plasmids carrying insert will form white colony whilst the negative ones will stay blue in the media containing X-gal (Figure 1). The blue colony was formed due to the activity of β galactosidase enzyme in the presence of X-gal. The cDNA library was inserted into multiple cloning sites upstream the lacZ gene (Figure 3). The inserted cDNA will disrupt the reading of this gene resulted in white colony. In addition to this assay, the presence of recombinant plasmid was also further confirmed by digesting the plasmids with *EcoRI*. pUC19 plasmid digested using the same restriction enzyme was used as negative control. From nine colonies screened, eight colonies showed other bands in addition to the original pUC19 with 2686 bp in size, suggesting that the insertion was successful (Sudjadi *et al.*, 2010). In this study among other recombinant plasmids which have various sizes of inserts, one of them, namely rMJC15310 has ~ 8kb in size, was cut into three fragmented inserts (Figure 2). In parallel to this assay, the proteins expressed in *E.coli* carrying the recombinant plasmids were isolated by sonication and then tested for the ability to cut supercoiled DNA. From several proteins tested, recombinant proteins rMJC15310, was chosen to be characterized further.

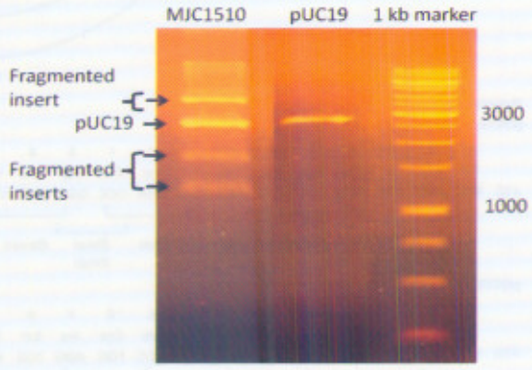

Figure 2. The DNA electrophoresis of rMJC15310 digested with EcoRI (Sudjadi *et al.*, 2010).

This recombinant protein cut supercoiled DNA. The ability of rMJC15310 to cut supercoiled DNA was optimized by increasing concentration of recombinant protein. Increasing amount of rMJC15310 showed a slight increase in the ability to cut supercoiled DNA, with more obvious data seen with 30 μg protein (Sudjadi *et al.*, 2010).

In order to determine whether the DNA cleavage was due to the activity of the recombinant protein, the expression of rMJC15310 was induced by adding IPTG then the ability of the proteins to cut supercoiled DNA was examined. Two concentration of proteins were used, 5 μg and 20 μg respectively.

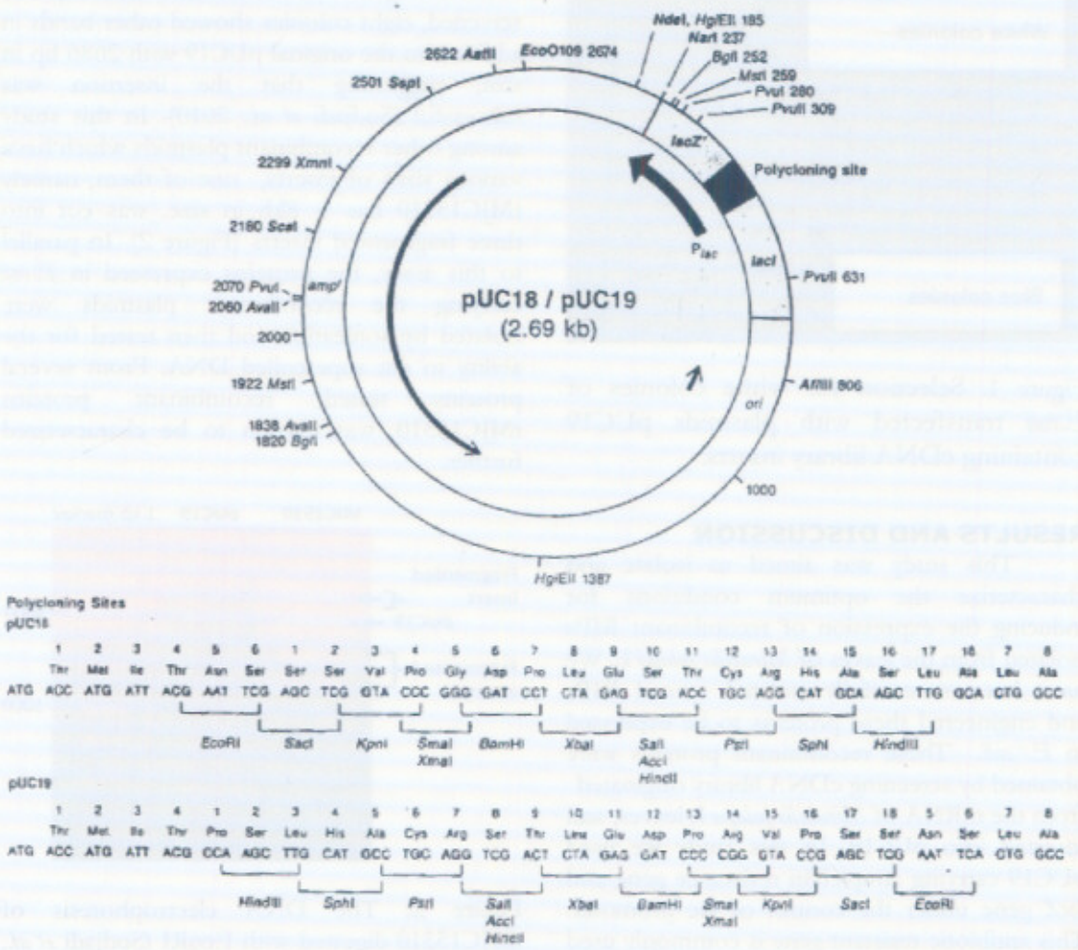

Figure 3. Vector cloning pUC18/19 (clontech).

As seen in figure 4 and 5, the addition of proteins isolated from the *E.coli* culture transfected with plasmid control did not resulted in cleaved supercoiled DNA, whilst the insertion of the recombinant protein rMJC15310 induced the cleavage. Inducing the expression of the recombinant protein with IPTG exhibited higher activity indicating that indeed the cleavage was due to the recombinant protein.

Many plants contain proteins having ability to inhibit protein synthesis like that of Ribosome Inactivation Proteins (RIPs). The biological and biomedical properties of these proteins have attracted scientists to explore and characterize further what RIPs actually can do. In this study we have successfully isolated RIPs from *Mirabilis jalapa* L leaves and engineered these proteins to be expressed in *E.coli*. Initially, the mRNA was isolated from the leaves of *Mirabilis jalapa* L and cDNA library was constructed. Using this approach, the library can be inserted to the vector and the recombinant products from this library can be screened for the ability to cut supercoiled DNA, one of the RIPs properties.

In parallel to this experiment, the white colonies were further grown and the proteins expressed were isolated and tested for the ability to cut supercoiled DNA. Besides RIPs activity on ribosomal RNA, RIPs have been reported to possess enzymatic activity by cleaving supercoiled double stranded DNA to become nick circular and linear DNA

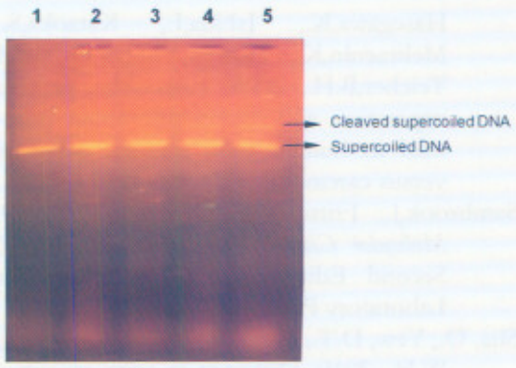

Figure 4. Gel electrophoresis of supercoiled DNA treated with rMJC15310 at 5 µg
1. negative control, 2. Protein isolated from *E.coli* tranfected with mock plasmid, 3. protein isolated from E.coli transfected with plasmid pUC19 control, 4. protein isolated from *E.coli* transfected with rMJC15310 (without IPTG), 5. protein isolated from *E.coli* transfected with rMJC15310 (with IPTG)

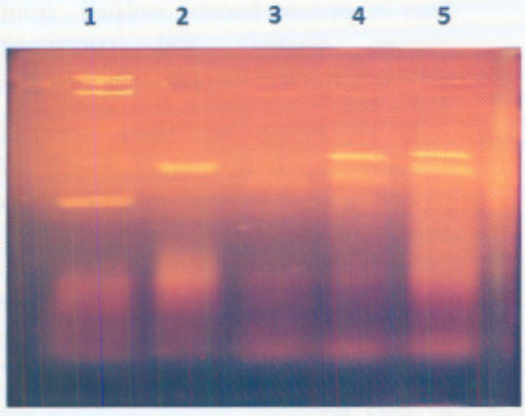

Figure 5. Gel electrophoresis of supercoiled DNA treated with rMJC15310 at 20 µg.
1. negative control (supercoiled pUC19); 2. Liniear pUC19; 3. pUC19 protein isolated from *E.coli* transfected with plasmid pUC19 control; 4. protein isolated from *E.coli* transfected with rMJC15310 (without IPTG); 5. protein isolated from *E.coli* transfected with rMJC15310 (with IPTG).

(Li *et al.*, 1991; Ling *et al*, 1994, Sismindari *et al.*, 1998). From many white colony screened we have been able to detect few colonies with this enzymatic property and picked up one to be characterized further. rMJC15310 was further tested by the increasing amount of proteins added in the test tubes containing pUC19. It was found that the extent of supercoiling was altered by increasing the amount of protein. However, from all the concentration used we failed to detect the lowest concentration of rMJC15310 which produced nick circular DNA.

In order to confirm that the enzymatic activity was due to the recombinant protein, the expression of rMJC15310 was induced by adding IPTG to the media and the RIPs activity was tested again. Adding IPTG to the media increased the activity of rMJC15310 product to cut supercoiled DNA. IPTG is a lactose analog commonly added to culture medium to induce the expression of the gene under the control of lac promoter (Lodish *et al.*, 2000). As the linier form increased, the supercoiled form disappear from the agarose gel. This data provides further support for the one of the enzymatic property of RIP imposed by rMJC15310.

CONCLUSION

rMJC15310, RIP isolated from the leaves of *Mirabilis jalapa* L., was engineered to be expressed in *E. coli*. This recombinant, having ~ 8kb insert, cut the supercoiled DNA into the linear form at the concentration as low as 5 µg and the activity was increased by inducing its expression with 0.4% IPTG.

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